Supplementary Material (ESI) for Chemical Communications

Supplementary Information

Bio-Reversible polyPEGylation

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1. Materials

Ethanethiol (99+%, ACROS), 3,3'-dithiodipropionic acid (99%, Aldrich), 2-mercaptothiazoline (98%, Aldrich), *N,N'*-dicyclohexylcarbodiimide (DCC, 99%, Sigma), 4-(dimethylamino) pyridine (DMAP, 99%, Aldrich), tetraethylene glycol (99%, Aldrich), *Micrococcus lysodeikticus* (Ml cell, Sigma) and lysozyme (from chicken egg white, Sigma) were used as purchased. 2-(Dimethylamino)pyridinium *p*-tolenesulfonate (DPTS)[1] and 4-cyano-4-(ethylthiocarbonothioylthio) pentanoic acid[2] were synthesized as previously described. 2,2'-Azobis(isobutyronitrile) (AIBN, 98%, Sigma-Aldrich) was recrystalized twice from acetone, dichloromethane (DCM, 99%, Ajax) was stored over calcium hydride and distilled before using.

Abbreviations: chain transfer agent (CTA); dichloromethane (DCM); molecular weight (MW); polydispersity index (PDI); 2,2'-azobis(isobutyronitrile) (AIBN) ; tetrahydrofuran (THF); 4-(dimethylamino)pyridine (DMAP); tris(2-carboxyethyl) phosphine hydrochloride (TCEP); gel permeation chromatography (GPC); molecular weight cut-off (MWCO); phosphate buffered saline (PBS); sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); reverse phase HPLC (RP HPLC); trifluoroacetic acid (TFA); *Micrococcus lysodeikticus* (Ml cell).

2. Measurement

Gel permeation chromatography (GPC) analyses of polymers were performed using tetrahydrofuran (THF) as the eluent (flow rate: 1.0 $mL.min^{-1}$) using a Shimadzu modular system comprising an auto injector, a Phenomene x 5.0 μm bead-size guard column (50 × 7.5 mm) followed by three Phenomenex 5.0 μm bead-size columns (10^5, 10^4, and 10^3 Å), and a differential refractive-index detector. The system was calibrated with polystyrene (PSt) standards with molecular weights of 200 to $10^6 g\ mol^{-1}$.

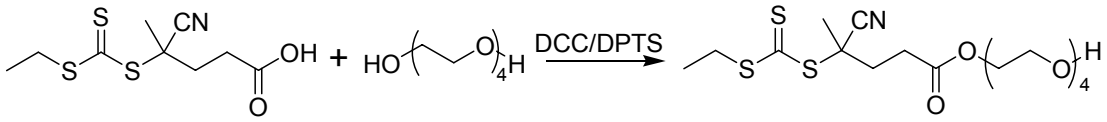
The UV-vis absorption spectra were recorded on a Cary 300 Scan spectrophotometer (Varian). ^1H NMR spectra were obtained using a Bruker AC300F (300 MHz) Spectrometer or a Bruker DPX300 (300 MHz) Spectrometer. Multiplicities were reported as singlet (s), broad singlet (bs), doublet (d), triplet (t), and multiplet (m). Mass spectra were obtained on a Finnigan LCQ Deca ion trap mass spectrometer (Thermo

Finnigan, San Jose, CA) equipped with an atmospheric pressure ionization source operating in the nebulizer-assisted electrospray mode. The instrument was calibrated in the *m*/*z* range 195-1822 Da using a standard containing caffeine, Met-Arg-Phe-Ala acetate salt (MRFA), and a mixture of fluorinated phosphazenes (Ultramark 1621) (all from Aldrich).

RP-HPLC characterization was carried out using a Finnigan MAT HPLC with a Luna 5μ C8 (2) column 150 × 4.60 mm (Phenomenex, UK). Gradient elution was carried out at a flow rate of 1.0 mL/min with a mobile phase A (99.9 % H_2O, 0.01 % TFA) and a mobile phase B (99.9 % acetonitrile, 0.01 % TFA). The gradient sequence (B) was: 10 – 75 % from 0 – 40 mins, 75 % from 40 – 45 mins, 75 - 10 % from 45-46 mins, 10 % from 46 – 56 mins. The sample was monitored at a UV absorbance of 280 nm. SDS-PAGE was carried out with 4-20 % Tris-HCl gels (Bio-rad, 1.0 mm x 10 well).

3. Methods

3. 1 2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl 4-cyano-4-(ethylthio carbono thioylthio) pentanoate (1, CTA 1)

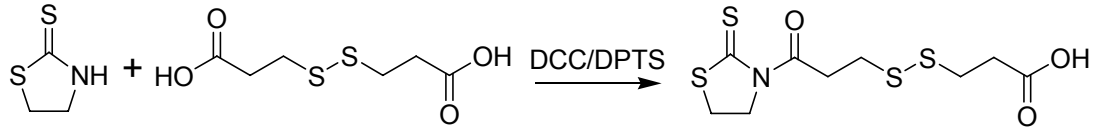


1 (1.50 g, 0.0057 mol), tetraethylene glycol (6.60 g, 0.034 mol) and DPTS (0.10 g) were dissolved in DCM (50 mL), DCC (1.30 g, 0.0063 mol) was added under a nitrogen atmosphere. The mixture was stirred at 20 °C for 6 h, filtrated to remove solid, washed with saturated NaCl aqueous solution (3 × 40 mL) and dried over $MgSO_4$. Volatiles was evaporated under vacuum and the crude was purified by column chromatography on silica gel (2.0 % methanol in DCM) to yield the product as a yellow oil (1.77 g, 70.6 %).

^{1}H NMR (300.18 MHz, $CDCl_3$)/ppm: 4.27-4.24 (m, 2H, $COOCH_2$), 3.71-3.58 (m, 14H, $C\underline{H_2}OH$, 3×CH_2OCH_2), 3.33 (q, J = 7.4 *Hz*, 2H, $SC\underline{H_2}CH_3$), 2.68-2.32 (m, 4H, CH_2CH_2CCN), 1.87 (s, 3H, CH_3CCN), 1.35 (t, J = 7.4 *Hz*, 3H, SCH_2CH_3) ^{13}C NMR (75.49 MHz, $CDCl_3$)/ppm: 216.89, 171.62, 119.11, 73.06, 72.65, 70.75, 70.41, 69.08,

64.21, 61.83, 61.68, 46.43, 33.87, 31.46, 29.79, 24.91, 12.86. IR (cm^{-1}): 2868, 1733, 1246, 1186, 1072, 803. ESI-MS: $M+Na^+$ expected (observed): 462.11 (462.12).

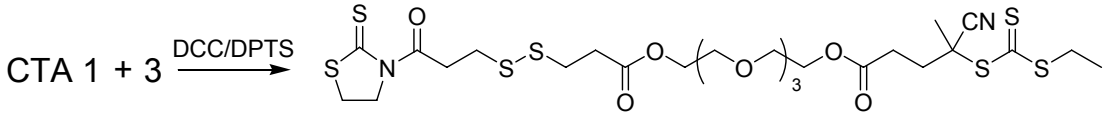
3. 2 3-((3-oxo-3-(2thioxothiazolidin-3-yl)propyl)disulfanyl)propanoic acid (2)



3,3'-dithiodipropionic acid (10.50 g, 0.05 mol), 2-mercaptothiazoline (2.98 g, 0.025 mol) and DPTS (0.10 g) were dissolved in DCM (150 mL), DCC (5.67 g, 0.028 mol) was added under nitrogen atmosphere. The mixture was stirred at 20 °C for 20 h, filtrated to remove solid and distilled under vacuum to remove solvent. The crude product was purified by column chromatography on silica gel (from DCM to 1.0 % methanol in DCM) to yield the product as a yellow powder (5.10 g, 65.5%).

^{1}H NMR (300.18 MHz, $CDCl_3$)/ppm: 4.58 (t, *J* = 7.5 *Hz*, 2H, NCH_2), 3.68 (t, *J* = 7.0 *Hz*, 2H, $CH_2(C=O)N$), 3.31 (t, *J* = 7.5 *Hz*, 2H, $NCH_2C\underline{H_2}$)), 3.01 (t, *J* = 7.0 *Hz*, 2H, CH_2COOH), 2.95-2.76 (m, 4H, CH_2SSCH_2). ^{13}C NMR (75.49 MHz, $CDCl_3$)/ppm: 201.76, 177.09, 172.82, 56.02, 38.85, 33.98, 33.26, 32.91, 28.56. IR (cm^{-1}): 2850, 1678, 1623, 1341, 1265, 1230, 1217, 1195, 1166, 1043. ESI-MS: $M+Na^+$ expected (observed): 333.97 (334.00).

3. 3 13,20-dioxo-20-(2-thioxothiazolidin-3-yl)-3,6,9,12- tetraoxa-16-17-dithiaicosyl 4-cyano-4-(ethylthiocarbonothioylthio)pentanoate (3, CTA 2)



CTA 1 (1.00 g, 0.0023 mol), 3 (0.64 g, 0.0021 mol) and DPTS (0.05 g) were dissolved in DCM (50 mL). DCC (0.52 g, 0.0025 mol) was added under nitrogen atmosphere. The mixture was kept stirring at 20 °C for 18 h, filtrated to remove precipitate and distilled to remove solvent under vacuum. The crude was purified by column chromatography on silica gel (1.0 % methanol in DCM) to yield the product as a yellow oil (1.10 g, 71.5%).

^{1}H NMR (300.18 MHz, $CDCl_3$)/ppm: 4.58 (t, J = 7.5 *Hz*, 2H, NCH_2), 4.27-4.24 (m, 4H, 2×$COOCH_2$), 3.71-3.64 (m, 14H, 3×CH_2OCH_2, $CH_2(C{=}O)N$), 3.37-3.28 (m, 4H, SCH_2CH_3, NCH_2CH_2), 3.00 (t, J = 6.80 *Hz*, 2H, SCH_2CH_2COO), 2.95-2.74 (m, 4H, CH_2SSCH_2), 2.68-2.32 (m, 4H, CH_2CH_2CCN), 1.87 (s, 3H, CH_3CCN), 1.35 (t, J = 7.4 *Hz*, 3H, SCH_2CH_3). ^{13}C NMR (75.49 MHz, $CDCl_3$)/ppm: 216.88, 201.75, 172.80, 171.79, 171.59, 119.11, 89.68, 70.72, 70.70, 69.17, 69.08, 64.25, 64.00, 56.03, 50.97, 46.44, 38.86, 34.18, 33.89, 33.28, 33.21, 31.48, 29.81, 28.56, 24.94, 12.87. IR (cm^{-1}): 2870, 1731, 1694, 1352, 1280, 1229, 1137, 1046, 860, 804. ESI-MS: $M+Na^+$ expected (observed): 755.07 (755.07).

3. 4 Synthesis of polyPEGMA

A typical polymerization procedure is described below:

PEGMA (M_n~475, 0.50 g, 1.05 mmol), CTA 2 (38.6 mg, 0.053 mmol) and AIBN (1.7 mg, 0.011 mmol) were dissolved in 4.0 mL of dioxnae. Aliquots were transferred to eight different vials, which were then sealed with rubber septa. Each vial was deoxygenated by purging with nitrogen for 30 min prior to placement in a preheated oil bath at 80 °C. The vials were taken out at 0.5, 1, 1.5, 2, 2.5, 4, 5 and 6 hours. Immediate cooling with an ice-water bath and exposure to air quenched the polymerizations. The monomer conversion for each polymerization sample was determined using ^{1}H NMR directly. After removing the volatiles from the polymerization mixtures under vacuum, the residues were re-dissolved in tetrahydrofuran (THF) for GPC analysis. The final polymers were collected after precipitation three times from THF to diethyl ether, and then dried under vacuum. CTA 1 was employed to prepared hydroxyl terminated polyPEGMA (polymer 1) by the same procedure.

The monomer conversion against the polymerization times, the molecular weight (MW) and PDI against the monomer conversion are shown in Figure 1S as below.

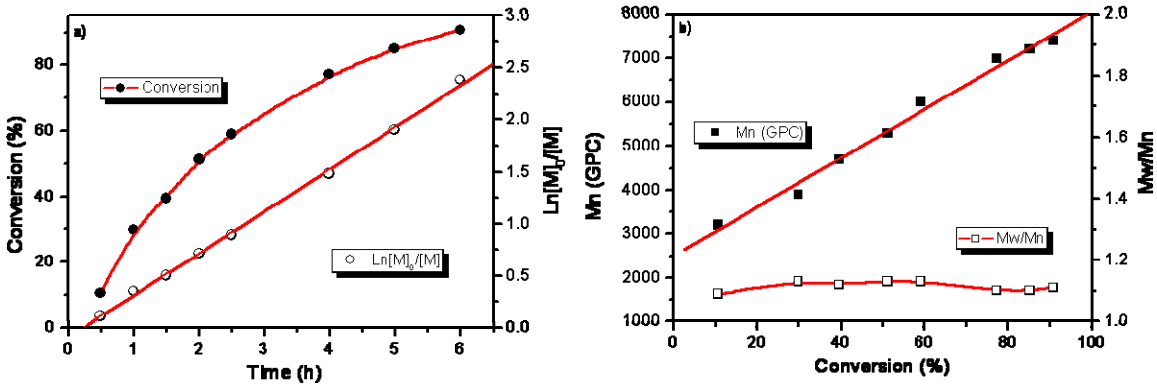


Figure 1S. Polymerization of $PEGMA_{475}$ using CTA 2 in Dioxane at 80 °C ($[M]_0$: [CTA]: [AIBN] = 20: 1: 0.2). a) Monomer conversions and the kinetic curve versus polymerization time; b) molecular weights (MW) and PDI of polymer versus monomer conversion.

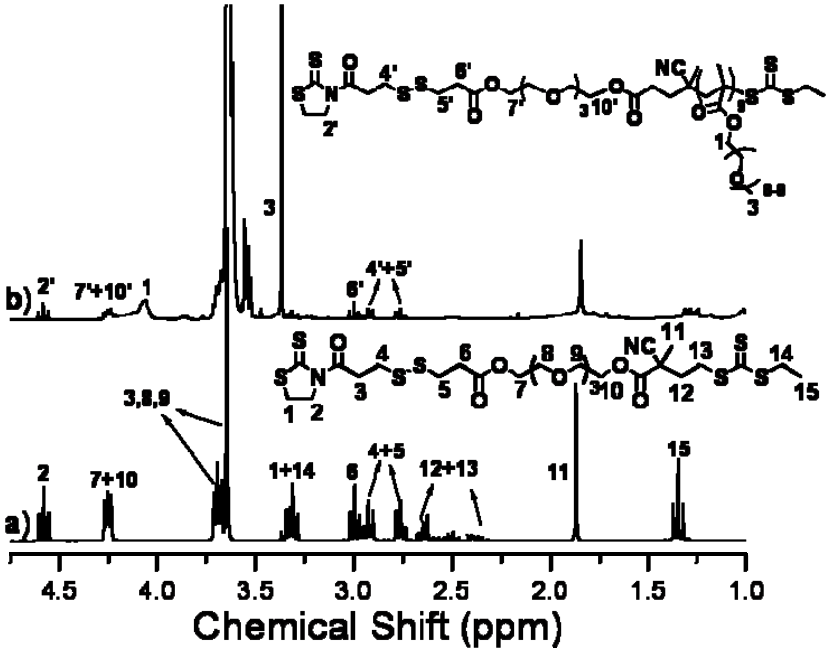


Figure 2S. ^1H NMR spectra of a) CTA and b) polymer 2.

3.5 Bio-activity test of the protein-polymer conjugates.

The bio-activities of the conjugates were tested using Ml cell as substrate and the operation was repeated three times to get mean average data.

16.8 mg of Ml cell was suspended in PBS buffer solution (90 mL, pH 7.0). An aliquot of suspension (3.0 mL) was transferred to a cuvette. The initial absorbance at wavelength 450 nm was defined as the baseline. Subsequently lysozyme solution (5.0 μL of 1.0 mg/mL in PBS buffer, pH 6.5) was added and the absorbance was measured every 15 seconds for 3 mins. The activity was calculated from the equation A (unit/mL) = -K/(0.001VD) where A is defined as relative lysozyme concentration, K is the slope of graph, V is the volume (mL) of sample solution and D is the dilution coefficient. The data were used as a control in the calculation of the retention of bio-activity of protein-conjugates as shown in Figure 3S. The activity analysis of the protein in control reaction (polymer 1: protein = 40: 1) was operated using same method (Fig 4S). The bioactivity of protein-polymer conjugates were tested using the same fashion as that for native lysozyme, that is, adding 5.0 μL of the conjugation solution to the Ml cell suspension (3.0 mL), followed by absorbance analysis (450 nm) as detailed above.

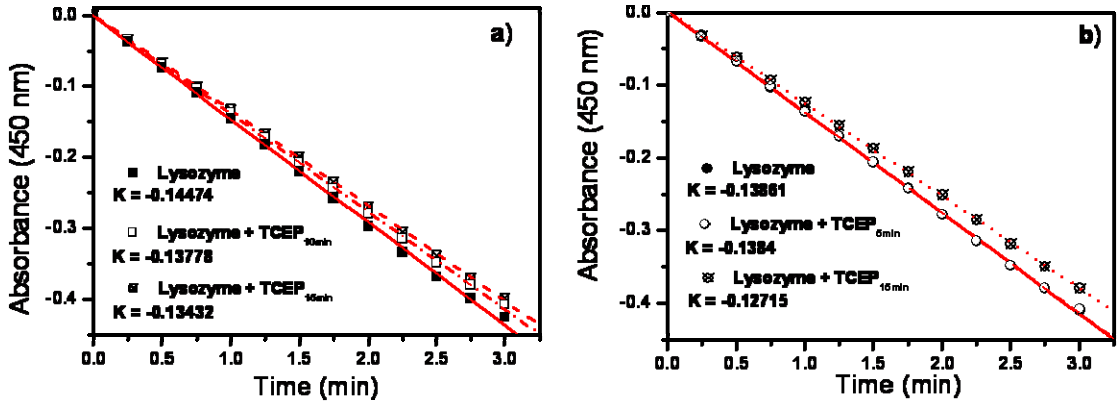


Figure 3S. Bioactivity test of native lysozyme before and after adding TCEP. a) pH 6.5, b) pH 7.0.

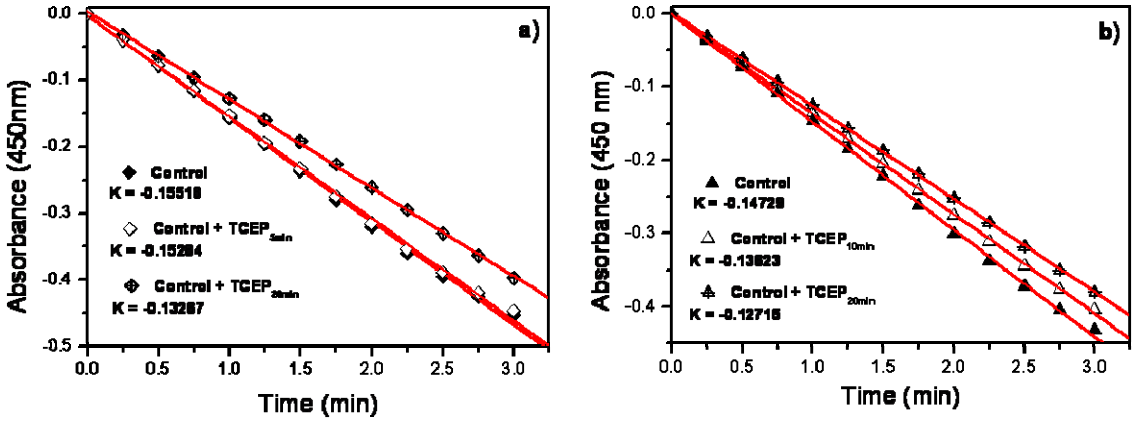


Figure 4S. Bioactivity test of control experiment (polymer 1 + protein) before and after adding TCEP. a) pH 6.5, b) pH 7.0.

3.8 Cleavage of conjugate with TCEP.

A typical procedure of protein-polymer conjugate cleavage under pH 7.0 was operated as follow:

Conjugation solution (190 μL, protein concentration: 1.0 mg/mL) was removed and TCEP solution (10.0 μL, 0.3 M) was added. The mixed solution (5.0 μ L, protein concentration: 0.95 mg/mL) was removed and the bioactivity was tested as described above every 5 mins, mean average data were recorded after three operations. After the bioactivity test, the remaining solution was diluted with 200 μL of distilled water and used for HPLC analysis directly. The RP-HPLC analysis is shown in Figure S5. The bioactivity test of released protein from conjugate prepared at pH 7.0 is displayed in Figure S6.

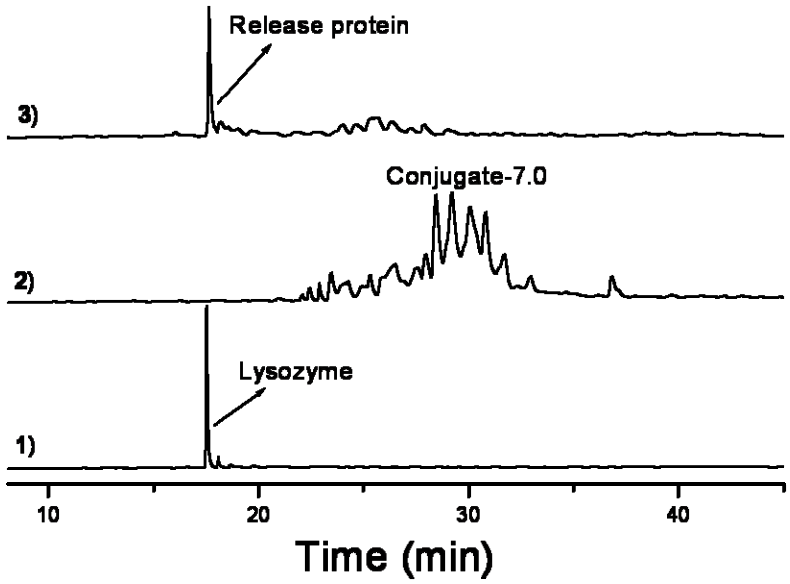


Figure S5. RP-HPLC curves of 1) native lysozyme, 2) lysozyme-polymer2 conjugate (pH 7.0) and 3) conjugate + TCEP.

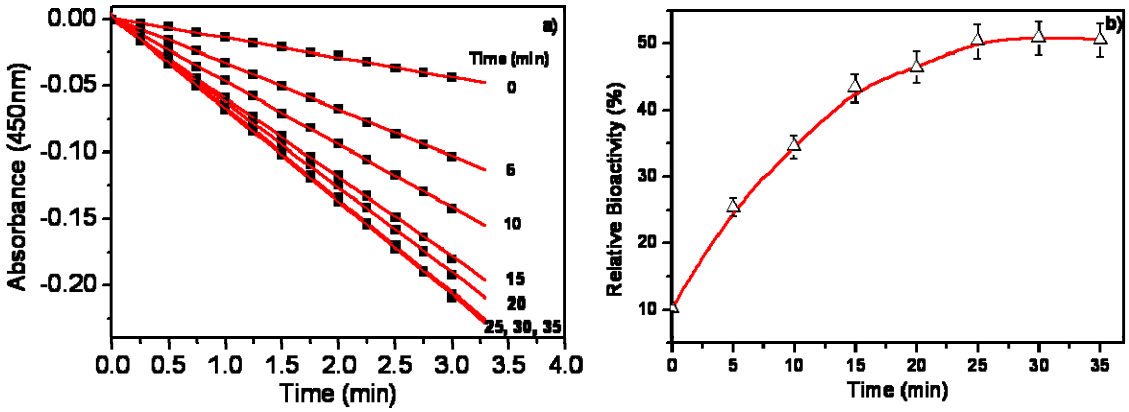


Figure S6. Bioactivity test of regenerated protein. a) conjugate$_{7.0}$ + TCEP, b) relative bioactivity of released protein to native lysozyme (mean averages ± SD three tests).

References:

1. H. Ihre, A. Hult, J. M. J. Frechet and I. Gitsov, *Macromolecules*, 1998, **31**, 4061-4068.
2. A. J. Convertine, D. S. W. Benoit, C. L. Duvall, A. S. Hoffman and P. S. Stayton, *J. Controlled Release*, 2009, **133**, 221-229.